Extracellular OCR Plate Assay Kit

Technical Manual

General Information

Oxygen is consumed mainly in the process of adenosine triphosphate (ATP) production by mitochondrial oxidative phosphorylation. Therefore, the oxygen consumption rate (OCR) of cells is an indicator of mitochondrial function. Cancer cells produce ATP using glycolysis, which is less efficient than oxidative phosphorylation. In immune cells, predominance of oxidative phosphorylation inhibits anti-tumor effects, whereas predominance of the glycolytic system promotes anti-tumor effects. The OCR of cells can be measured as an indicator of their energy metabolism.

The Extracellular OCR Plate Assay Kit enables measurement of the OCR of cultured cells. The kit Includes an Oxygen Probe, which increases phosphorescence intensity when the oxygen concentration in the medium decreases, and Mineral Oil to block the influx of oxygen from the air. The kit does not require any special equipment, media, or plates and can be used with a general-purpose fluorescence microplate reader and microplates.

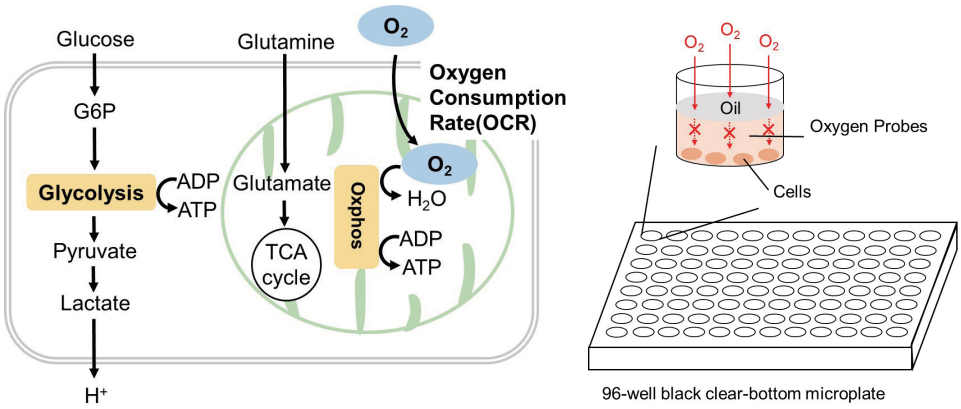


Figure 1. Principle of Extracellular OCR Plate Assay Kit

Kit Contents

Oxygen Probe	110 µl×1
Mineral Oil	10 ml×1

Storage Conditions

Store at −20 °C

Required Equipment and Materials

- Microplate reader with temperature control
- 96-well black clear-bottom microplate
- 20−200 µl multichannel pipette
- 100−1000 µl, 20−200 µl and 2−20 µl micropipettes
- 1.5-ml microtube
- Conical tube
- Heating block or water bath

Precautions

- Equilibrate reagents to room temperature prior to use.
- Briefly centrifuge the Oxygen Probe tube before opening it to remove all content from the walls of the tube and the inside of the cap.
- Analysis of samples in triplicate is recommended for accuracy.
- Set the microplate reader temperature at 37℃ before measurement.
- Pre-warm the Mineral Oil and sample solutions to around 37℃ in a heating block or water bath.
- Following cell numbers are recommended in case using a 96-well microplate.
  Suspension cells: 200,000−400,000 cells/well, Adherent cells: 30,000−50,000 cells/well

Preparation of Solutions

Preparation of a working solution

※ Please use the serum-containing medium in the experiment.

(1) Add medium to a conical tube.

(2) Add Oxygen Probe to the medium (see Table 1).

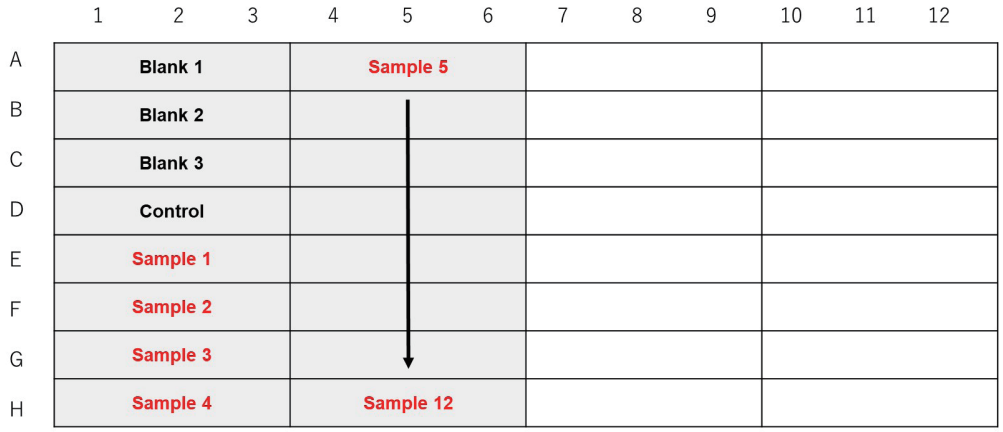
※ The working solution is light-sensitive. Prepare the solution just before use and protect it from light by covering it with aluminum foil. Please use up the working solution within that day.

Table 1. The required amount of working solution by sample number

	15 wells	30 wells	45 wells	60 wells	75 wells	90 wells
Oxygen Probe	16.5 µl	33 µl	49.5 µl	66 µl	82.5 µl	99 µl
Medium	1483.5 µl	2967 µl	4450.5 µl	5934 µl	7417.5 µl	8901 µl

〈Suspension cells〉

When testing sample type or concentration in the same cell line and the number of cells.



When testing different cell lines.

When testing different number of cells.

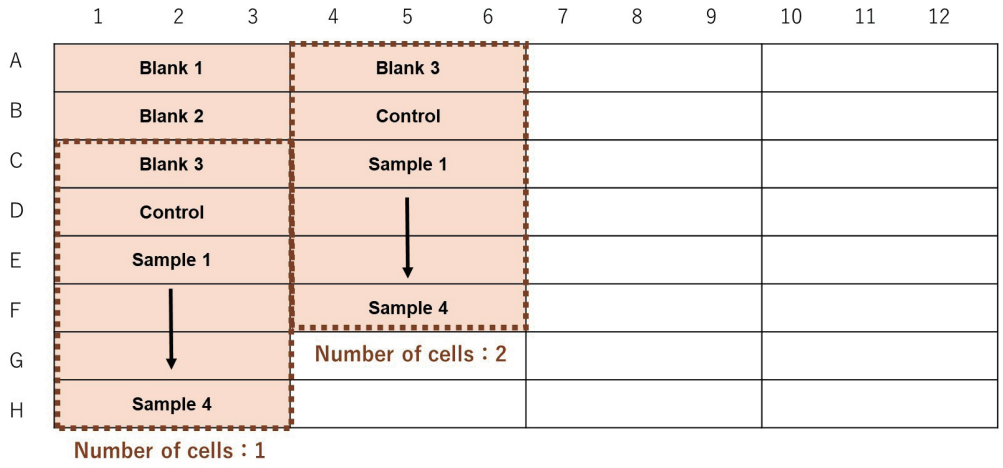


Figure 2. Example assay plate formats (n=3)

Table 2. The required amount of samples and reagents for each well

	Blank 1	Blank 2	Blank 3	Control	Sample
Medium	110 µl	10 µl	10 µl	10 µl	–
working solution	–	100 µl	–	–	–
Cells (suspended in medium)	–	–	100 µl	–	–
Cells (suspended in working solution)	–	–	–	100 µl	100 µl
Sample solution	–	–	–	–	10 µl
Mineral Oil	1 drop	1 drop	1 drop	1 drop	1 drop

※ Please use the serum-containing medium in the experiment.

(1) Suspend cells ($2.0-4.0\times10^6$ cells/ml) in the medium for Blank 3 and in working solution for the Control and Samples (see Table 2). Seed them (100 µl) in a 96-well black clear-bottom microplate ($2.0-4.0\times10^5$ cells/well) (see Figure 2).

※ Triplicate measurements are recommended to obtain accurate data.

(2) Add 100 µl of medium to Blank 1 and 100 µl of working solution to Blank 2 (see Figure 2).

(3) Incubate the microplate for 30 minutes in a microplate reader (37 °C).

(4) Add 10 µl of medium to Blank 1, Blank 2, Blank 3, and the Control (see Figure 2).

※ Pre-warm the medium to around 37 °C in a heating block or water bath.

(5) Dilute a sample with a medium and add 10 µl to the Samples (see Figure 2).

※ Pre-warm the diluted sample solution to around 37 °C in a heating block or water bath.

(6) Immediately after adding the sample solution, add 1 drop of Mineral Oil to all wells.

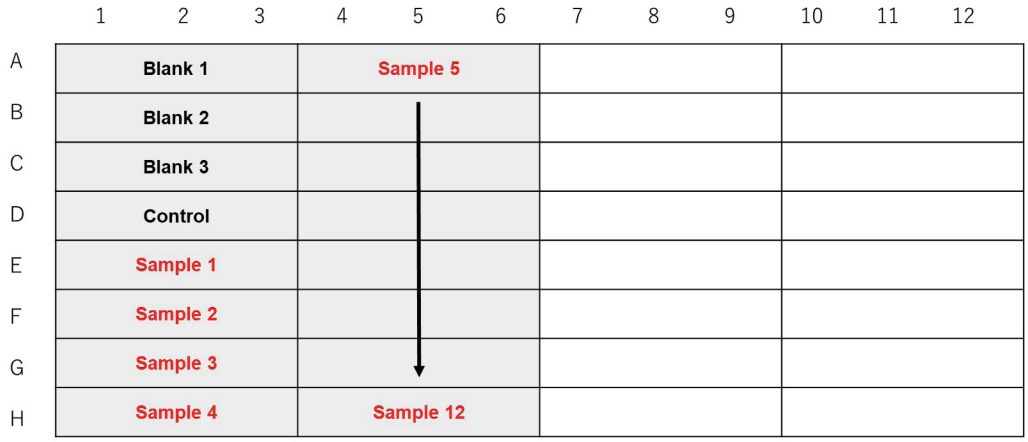
※ Pre-warm Mineral Oil to around 37 °C in a heating block or water bath.

(7) Incubate the microplate for 5 minutes in the microplate reader (37 °C).

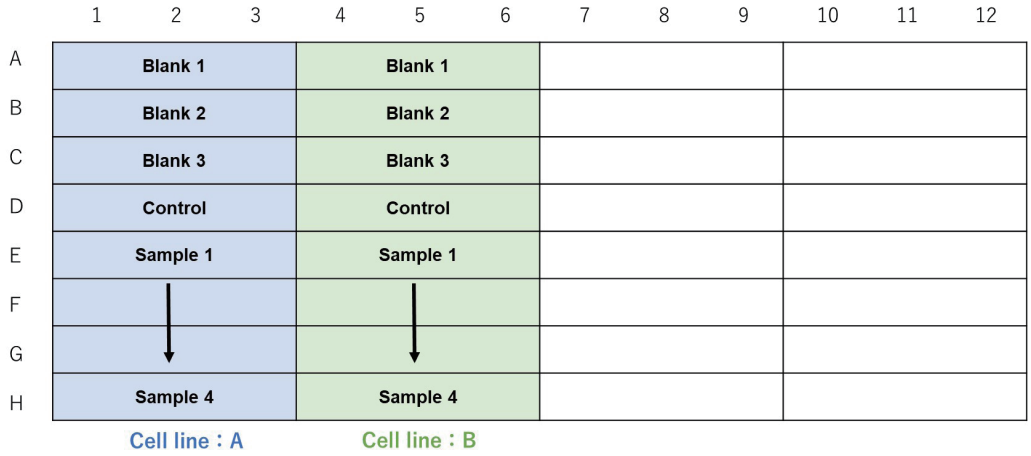
(8) Measure the kinetics using the microplate reader (recommended filter settings: Ex: 500 nm, Em: 650 nm, 10-min interval for 200 min, Bottom reading).

〈Adherent cells〉

When testing sample type or concentration in the same cell line and the number of cells.



When testing different cell lines.



When testing different number of cells.

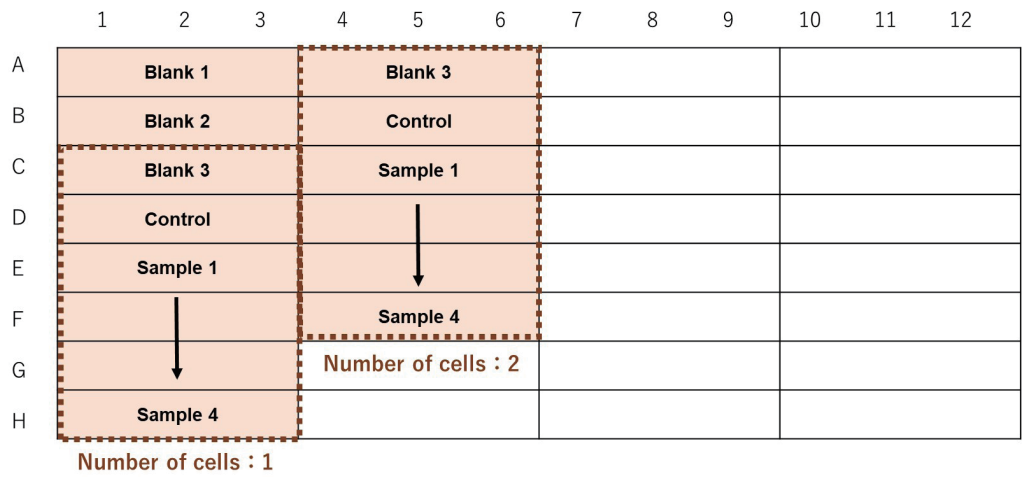


Figure 3. Example assay plate formats (n=3)

Table 3. The required amount of samples and reagents for each well

	Blank 1	Blank 2	Blank 3	Control	Sample
Medium	110 µl	10 µl	110 µl	10 µl	–
working solution	–	100 µl	–	100 µl	100 µl
Sample solution	–	–	–	–	10 µl
Mineral Oil	1 drop	1 drop	1 drop	1 drop	1 drop

※ Please use the serum-containing medium in the experiment.

(1) Seed cell suspension 100 µl ($3.0-5.0\times10^5$ cells/ml) in a 96-well black clear-bottom microplate for Blank 3, the Control, and Samples (see Figure 3).

※ Triplicate measurements are recommended to obtain accurate data.

※ If the normalization of the number of cells per well using nuclear staining (Hoechst 33342) is needed after the OCR measurement, please seed cells to the calibration wells. Please refer to the Q&A section about the example for adherent cells on website.

(2) Culture overnight in an incubator (37 °C, 5% CO_2).

(3) Remove the medium from the wells, taking care not to peel off the cells.

(4) Add 100 µl of medium to Blank 1 and Blank 3, and 100 µl of working solution to Blank 2, the Control, and Samples (see Figure 3).

(5) Incubate the microplate for 30 minutes in a microplate reader (37 °C).

(6) Add 10 µl of medium to Blank 1, Blank 2, Blank 3, and the Control (see Figure 3).

※ Pre-warm the medium to around 37 °C in a heating block or water bath.

(7) Dilute a sample with a medium and add 10 µl to the Samples (see Figure 3).

※ Pre-warm the diluted sample solution to around 37 °C in a heating block or water bath.

(8) Immediately after adding the sample solution, add 1 drop of Mineral Oil to all wells.

※ Pre-warm Mineral Oil to around 37 °C in a heating block or water bath.

(9) Incubate the microplate for 5 minutes in the microplate reader (37 °C).

(10) Measure the kinetics using the microplate reader (recommended filter settings: Ex: 500 nm, Em: 650 nm, 10-min interval for 200 min, Bottom reading).

(11) Calculate the OCR from the intensity using the Excel sheet downloaded from the E297 product page.

Analysis

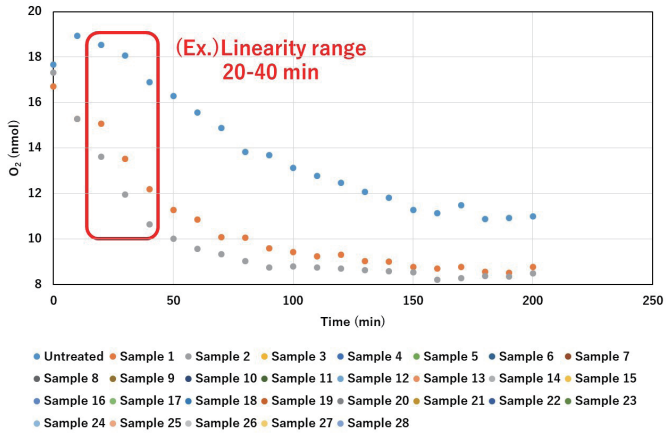
※ This calculation sheet is based on the experimental conditions as described in this product manual (i.e., well conditions, temperature, and liquid volume).

(1) Calculate the average value of intensity for each well condition at each time point of the measured data. Enter them in the corresponding orange cells in the Excel sheet (rows 3-34).

Time (min)	0	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200
Blank 1	118.5	124.25	121	114.25	117.5	118.75	118.5	122	118.75	120	115.5	114.25	123.75	114.5	120.75	119.5	124.5	122.75	118.25	112.25	120.75
Blank 2	2580.5	2355.75	2268.25	2298	2271	2285	2272.5	2258.25	2241.75	2267.75	2254.5	2226.75	2287	2253.75	2250	2214.5	2257.75	2256.5	2221.25	2193	2225.75
Blank 3	115	114.75	113	121.5	116	108	121.25	114	113.25	112.25	122.25	118	116.25	116.75	116.5	108	117.75	121.25	111.5	112.75	111.25
Control	2786	2655	2692	2749.5	2873	2938.5	3043.25	3128.5	3281	3300.5	3400	3457.5	3507.25	3578.75	3628.5	3725	3760.5	3696.25	3809.75	3796.75	3784.75
Sample 1	2894	3075.75	3102.25	3336	3557	3722.75	3821	3985.5	3988.25	4097	4147.75	4193.25	4172	4244.5	4249.25	4303.25	4331	4315.75	4366.25	4382.25	4309.5
Sample 2	2824	3073.25	3314	3604.25	3859.5	3995.25	4114	4161.25	4242.25	4316.5	4310	4320.25	4332.5	4352.5	4363	4368.5	4469.5	4455.75	4415	4428.5	4385.5

(2) From the visual graph data, determine a signal shared range of times that best describes linearity for as many well conditions as possible.

(3) Using the O_2 (nmol) and times (min) within the time range selected in (2), calculate the the OCR (nmol/min) per well. The example below uses the 20-40-minute range (cells D70 to F70).



O_2 (nmol)

Time (min)	0	10	20	30	40	50	60
Untreated	17.66835	18.92841	18.54165	18.06891	16.90471	16.28885	15.56545
Sample 1	16.71696	15.27071	15.06201	13.5292	12.19024	11.27684	10.86125
Sample 2	17.32495	15.28937	13.61499	11.96244	10.6547	10.00876	9.5643

Enter the following formula in the orange boxes below,
"= -SLOPE("known y", "known x")"
※ known y: Range of O_2 (nmol) over which linearity is obtained.
known x: Range of time (min) over which linearity is obtained.
Equations for the example above would be:
Untreated: "= -SLOPE(D71:F71,D70:F70)"
Sample 1: "= -SLOPE(D72:F72, D70:F70)"

OCR (nmol/min)→(pmol/min)

Untreated	0.081847	81.84661
Sample 1	0.143589	143.5886
Sample 2	0.148015	148.0148

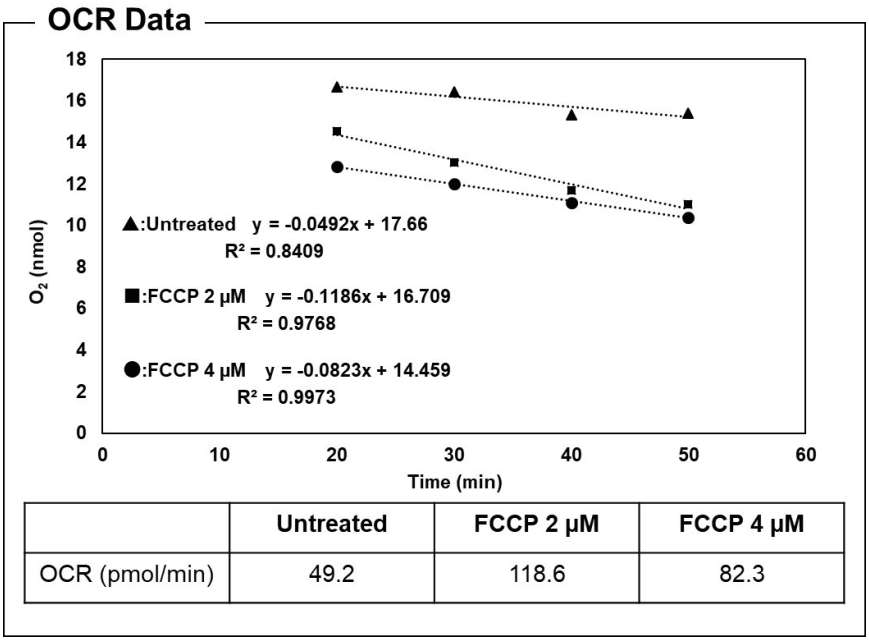
(4) When the formula above is entered, the units for OCR will automatically be converted from nmol/min to pmol/min in the adjacent column.

※ If you need the OCR normalized by cell number, perform a hemocytometer analysis or nuclear staining.

Experimental Example

Changes in OCR in HepG2 cells on treatment with mitochondrial uncoupler FCCP.

(1) HepG2 cells (5.0×10^5 cells/ml) were diluted in medium (DMEM containing 10% fetal bovine serum and 1% penicillin-streptomycin) and seeded in a 96-well black clear-bottom microplate (5.0×10^4 cells/well).
(2) The cells were cultured overnight in an incubator (37 °C, 5% CO_2).
(3) The medium was removed from the wells.
(4) DMEM containing 10% FBS (100 µl/well) or working solution (100 µl/well) was added to each well of the 96-well black clear-bottom microplate.
(5) The microplate was incubated at 37 °C for 30 minutes in a microplate reader.
(6) Blank 1, Blank 2, Blank 3, Control: DMEM containing 10% FBS (10 µl/well) and Sample: FCCP diluted in DMEM containing 10% FBS (10 µl/well) was added to the wells.
(7) Mineral Oil (1 drop) was added to all wells.
(8) The microplate was incubated at 37 °C for 5 minutes in the microplate reader.
(9) Kinetics were measured using the microplate reader (Ex: 500 nm, Em: 650 nm, 10-min interval for 200 min, Bottom reading).
(10) OCR was calculated from intensity using the Excel sheet.



	Untreated	FCCP 2 µM	FCCP 4 µM
OCR (pmol/min)	49.2	118.6	82.3

Figure 4. The OCR was increased by 2 µM FCCP treatment compared with untreated cells and then decreased by treatment with 4 µM FCCP compared with 2 µM FCCP.

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